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Gram Staining Apparatus for Space Station Applications

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A self-contained, portable Gram staining apparatus (GSA) has been developed for use in the microgravity environment on board the Space Station Freedom. Accuracy and reproducibility of this apparatus compared with the conventional Gram staining method were evaluated by using gram-negative and gram-positive controls and different species of bacteria grown in pure cultures. A subsequent study was designed to assess the performance of the GSA with actual specimens. A set of 60 human and environmental specimens was evaluated with the GSA and the conventional Gram staining procedure. Data obtained from these studies indicated that the GSA will provide the Gram staining capability needed for the microgravity environment of space.

Space Station Freedom is NASA's next initiative in establishing a permanent manned presence in space. Many practical challenges must be successfully met. Illnesses are likely to occur on Space Station Freedom, and the appropriate diagnostic and therapeutic equipment and procedures must be developed. The Gram stain procedure is a cornerstone of diagnostic microbiology; however, the handling of liquids in the microgravity conditions of space are especially troublesome. That is, in the absence of gravity, liquids do not fall to the bottom of a test tube nor are bubbles eliminated by rising to the top of the liquid. Special precautions must be exercised to contain liquids to prevent contamination of the crew's living and working quarters. Almost all common activities are several times more difficult to perform in the space environment. This study demonstrates the considerations necessary to adapt a simple microbiological procedure such as the Gram stain for use in space.

Earlier efforts to provide an effective in-flight staining technique resulted in a complex Gram stainer that was included in the in-flight medical support system for the Skylab missions (1, 3). This equipment worked well as long it was in constant use, but malfunctions occurred following long periods of nonuse (5, 8, 9).

In view of the difficulties experienced with the Gram stainer developed for Skylab, it became necessary to design a more reliable apparatus for staining smears in flight. It was recognized that, to be useful in the environment of Space Station Freedom, a staining device had to meet the following requirements: perform well under microgravity conditions; be self-contained, compact, and lightweight; require little or no spacecraft power; reduce reagent volumes to a minimum; accommodate a variety of staining procedures; and be simple in design and easy to operate to reduce the possibility of malfunction. Thus, a Gram staining apparatus (GSA) was developed in our laboratory from a series of evolutionary in-house designs. This equipment performed well during microgravity compatibility testing onboard NASA's KC-135 aircraft, in which 30-s periods of simulated weightlessness are achieved by flying in a parabolic flight path.

This report discusses the design of the GSA and the results of testing during a double-blind study comparing the staining capabilities of the GSA with those of the conventional procedure. The study was performed in two phases. Phase 1 was a comparison of the two methods that used 48 slides of pure culture bacteria. Phase 2 was a comparison of the two methods that used 120 slides of human and environmental specimens.

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MATERIALS AND METHODS

Design and initial procedures. The GSA consists of a slide-holding device that provides a capillary path across the face of a biological smear on a slide (Fig. 1). The Gram stain reagents were prepared by filling four 3-ml syringes with at least 2.0 ml of the following commercially available reagents: syringe 1, Gram's crystal violet (catalog no. 3329-75; Difco Laboratories); syringe 2, Gram's iodine (catalog no. 3331-75; Difco); syringe 3, decolorizer (ethyl alcohol, 95%); and syringe 4, Gram's safranin (catalog no. 3332-75; Difco). In addition, a timer and two 50-ml syringes, each filled with 50 ml of tap water, are required (Fig. 2).

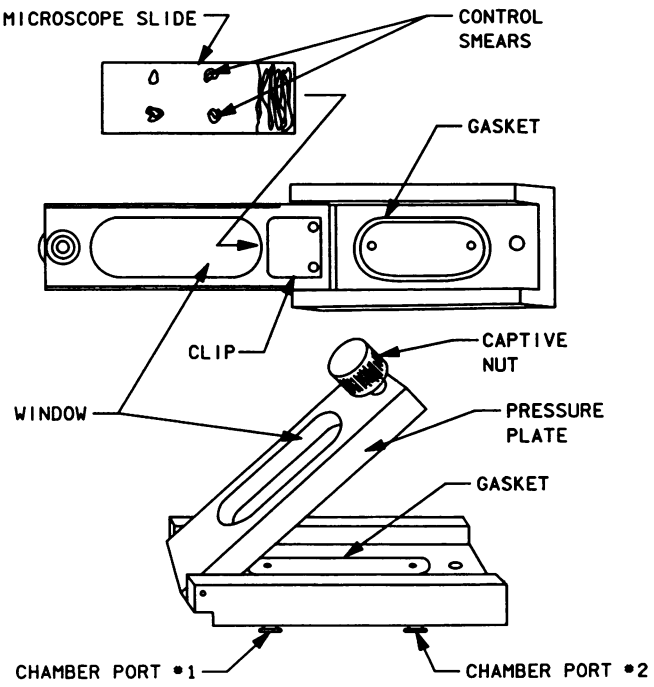

FIG. 1. GSA.

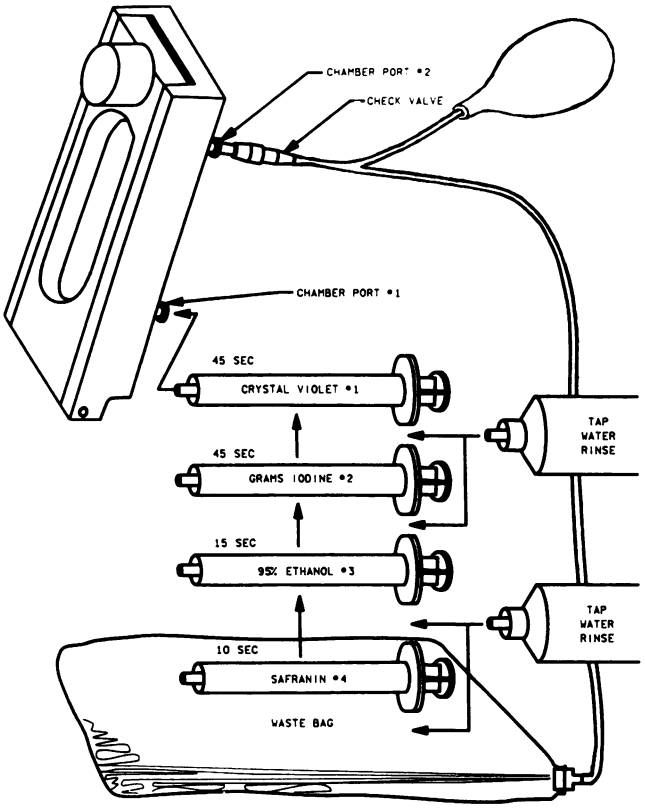

FIG. 2. Procedure for Gram stain.

GSA staining procedures. The slides in this study were heat fixed over a flame, but in the Spacelab environment the slides can be heat fixed on a hot plate before placing them in the chamber. The heat-fixed slide is placed with the frosted section under the hinged end of the GSA, positioning the smears inside the chamber. When the pressure plate is closed over the gasket as shown in Fig. 1, it is tightened over the gasket by using the captive nut. The tubing exiting at port 2 is attached to a waste bag (Fig. 2). Squeezing the bulb located distal to the check valve at port 2 (Fig. 2) creates a vacuum in the line when a syringe is attached to port 1. This vacuum aids in filling the chamber easily at port 1, and in emptying the chamber after removal of the syringe, in the microgravity environment. Syringe 1 is attached to chamber port 1, and the GSA is held vertically with the captive nut on top. The capillary chamber is filled with stain from syringe 1 at port 1, and the timer is set for 45 s. The syringe at port 1 is then replaced with a 50-ml syringe containing tap water. After the timed interval, the capillary chamber is purged with tap water from the large syringe. The tap water is removed by squeezing the bulb, and when the syringe is removed, water empties into the waste bag automatically. A check valve at port 2 ensures that the liquid flows from port 1 to port 2. This purge procedure is repeated three or four times until the solution in the chamber appears clear. The above procedures are then repeated with syringes 2, 3, and 4 at port 1, with exposures of 45, 15, and 10 s, respectively. The staining chamber is opened by unscrewing the captive nut, and the stained slide is removed. The stained slide is blotted with care so that the smear is not wiped off.

Conventional Gram stain technique. Heat-fixed slides are placed on a staining rack and flooded with Gram's crystal violet. After 1 min of contact time, the slide is rinsed with tap water until the unbound stain is washed off. The slide is then drained and flooded with Gram's iodine. After 1 min of contact with the iodine, the slide is again rinsed and drained. Holding the slide in a slanted position with forceps, 95% ethyl alcohol is poured on the slide until color ceases to run off the smear. (This usually requires 30 s to 1 min.) The slide is again rinsed with water and drained. The slide is counterstained by flooding with Gram's safranin for 30 s and then rinsed with water. The excess water is removed and the smear is blotted.

Specimens. The specimens for the first phase of the study were prepared from the pure-culture microorganisms listed in Table 1. Specimens for the second phase of the study consisted of two sets of slides prepared from six different sources. Ten slides for each set were made from each source. For example, if the first source was throat swabs, duplicate slides from each of 10 different throat swabs were made. One set was stained by using the GSA, and the other set was stained by the conventional method for comparison. Each slide contained a smear from one of the six different sources and two control smears, one for gram-positive and one for gram-negative bacteria. Two sets of 10 slides each were obtained from the following normal human sources: throat swabs, nose swabs, oral cavity swabs, and urine samples. Clinical specimens were obtained from hospital patients. Two sets of 10 slides each were made from the following clinical specimens: one urine, one abscess-pus, one gastric lavage, one stool, two sputum, two throat swabs, and two bronchial aspirates. The sixth source, environmental samples, consisted of two sets of 10 slides each made from the following areas: one stair well, one plant soil, one yogurt, one spoiled meat, two air vents, two urinals, and two sinks.

Method of evaluation. In phase 1, three sets of pure culture slides stained by three different technologists were pooled and prepared for the double-blind study (Fig. 3). The results were based on 128 data points: data from the reading of the slides by four technologists × 4 smears per slide = 16 data points per slide × 8 slides per set = 128 data points per set, calculated as the percent agreement with the expected

TABLE 1. Bacterial cultures examined for comparative evaluation

Species	Strain	% Correct interpretation[a]	
		GSA	Conventional
Bacillus subtilis		92	92
Enterobacter aerogenes	ATCC 13048	100	83
Escherichia coli	ATCC 25922	100	100
Klebsiella pneumoniae	ATCC 13883	92	100
Micrococcus sp.		100	100
Proteus mirabilis	ATCC 7002	100	100
Proteus vulgaris	ATCC 13315	92	92
Pseudomonas aeruginosa	ATCC 27853	100	100
Salmonella typhi	ATCC 14028	100	100
Serratia marcescens	ATCC 8100	100	100
Staphylococcus aureus	ATCC 29213	100	100
Staphylococcus epidermidis	ATCC 12228	100	100
Staphylococcus xylosus	ATCC 29971	92	83
Streptococcus faecalis[b]	ATCC 19433	100	100
Streptococcus faecium[b]	ATCC 6056	100	83
Streptococcus pneumoniae	ATCC 27336	100	67
Positive control		100	92
Negative control		83	59

[a] Frequency of correct interpretation of Gram reaction data expressed as percent correct interpretation from 12 observations.

[b] These two species have been transfered to the genus *Enterococcus*.

TABLE 2. Comparison of methodologies with reference to five parameters of staining quality from six clinical and environmental sources

Source	No. of slides in acceptable range (n = 10)[a]									
	Decolorization		Staining intensity		Stain precipitation		Uniformity of stain		Morphological detail	
	GSA	Conventional	GSA	Conventional	GSA	Conventional	GSA	Conventional	GSA	Conventional
Throat										
Test	10	10	10	10	10	10	10	10	10	10
Control	10	9	10	10	10	10	10	10	10	10
Oral cavity										
Test	9	10	10	10	10	10	10	10	10	10
Control	8	9	10	10	10	10	10	10	10	10
Urine										
Test	10	9	10	9	10	10	10	10	10	10
Control	10	8	10	10	10	10	10	10	10	10
Nasal										
Test	10	10	10	10	10	10	10	10	10	10
Control	10	7	10	10	10	10	10	10	10	10
Environmental										
Test	9	10	10	10	10	10	10	10	10	10
Control	9	7	10	10	10	10	10	10	10	10
Clinical										
Test	10	10	10	10	9	10	10	10	10	10
Control	10	7	10	10	9	10	10	10	10	10

[a] After normalization by two methods.

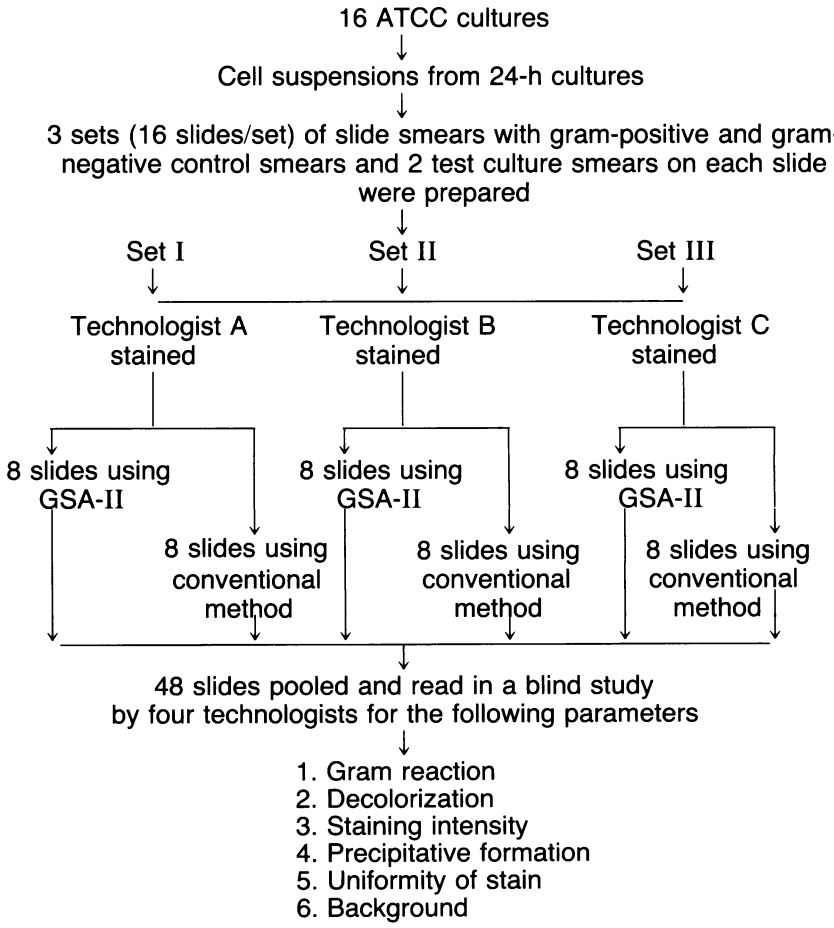

FIG. 3. Methodology of evaluation.

TABLE 3. Evaluation of cellular content of ten slides from each of six different sources[a]

Source and slide no.	GSA	Conventional	No. of different cells seen in each pair of slides
Throat			
1	4	4	8
2	2	**4**	7
3	**3**	2	7
4	**3**	2	6
5	2	2	8
6	3	**4**	5
7	3	**4**	6
8	3	**4**	8
9	4	4	7
10	3	**5**	10
Total[b]	2	5	
Oral			
1	3	3	11
2	2	2	6
3	1	**3**	11
4	3	3	9
5	4	4	8
6	3	3	10
7	4	4	8
8	3	**5**	10
9	**4**	3	10
10	**4**	3	9
Total	2	2	
Urine			
1	1	**3**	6
2	2	2	3
3	**4**	3	7
4	1	1	2
5	**1**	0	5
6	**2**	1	3
7	3	**5**	7
8	3	3	4
9	**5**	2	8
10	0	0	4
Total	4	2	
Nasal			
1	**5**	4	9
2	**3**	2	6
3	**5**	4	8
4	2	2	9
5	2	2	7
6	1	**2**	6
7	5	5	7
8	1	1	5
9	2	**3**	9
10	**3**	2	7
Total	4	2	
Environmental			
1	0	**1**	6
2	**2**	1	3
3	4	4	8
4	1	**2**	3
5	2	2	4
6	1	**2**	5
7	**2**	1	6
8	**2**	1	4
9	**4**	3	6
10	**2**	1	4
Total	5	3	
Clinical			
1	4	**5**	7
2	3	**4**	7
3	2	**3**	8
4	4	4	7
5	2	**4**	7
6	4	4	8
7	**5**	3	8
8	**6**	5	8
9	2	**3**	7
10	**4**	2	8
Total	3	5	

[a] Numbers in boldface indicate highest number of cells with at least three observations per set.
[b] Total number of sets with the highest number of observations.

results (100% being total agreement). The final results are an average of the three sets (see Fig. 4).

In phase 2, four technologists read two sets of 60 slides each from six different sources (Table 2) and recorded data on the same variables as in phase 1 for staining performance. A comparison was made of the two staining methods on the basis of these variables. Data for the aforementioned five variables were normalized by two methods. The data for the five variables were first graded on a scale of 0 to 5+ for values from ideal to intensely stained and 0 to 5− for values from ideal to weakly stained. For normalization, an acceptable range of 0 to ±2 was chosen. Also, the interpretations of

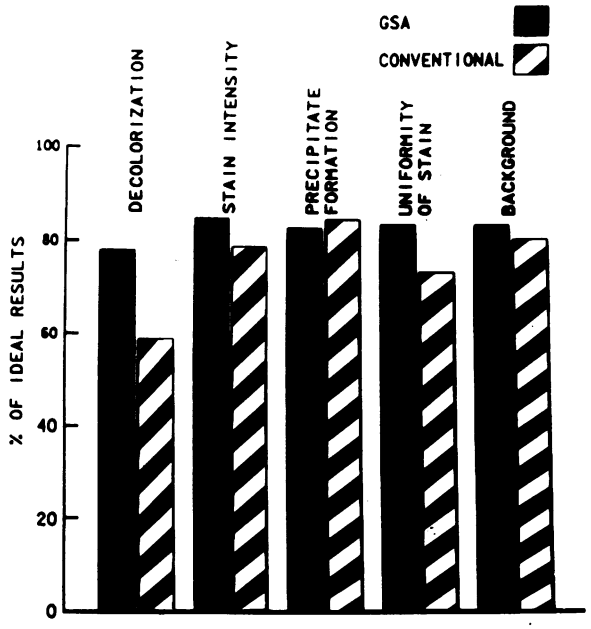

FIG. 4. Detailed comparison of methodologies with reference to five parameters of staining quality.

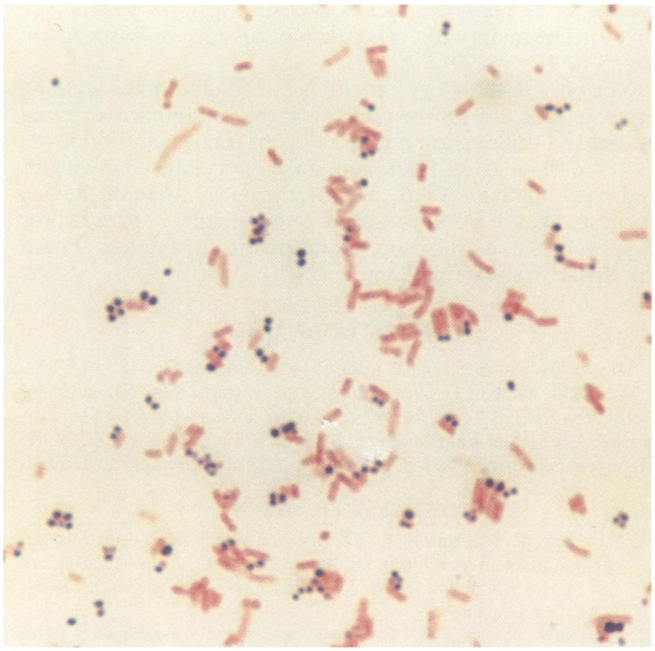

FIG. 5. Gram stain of gram-positive cocci and gram-negative rods, using the GSA.

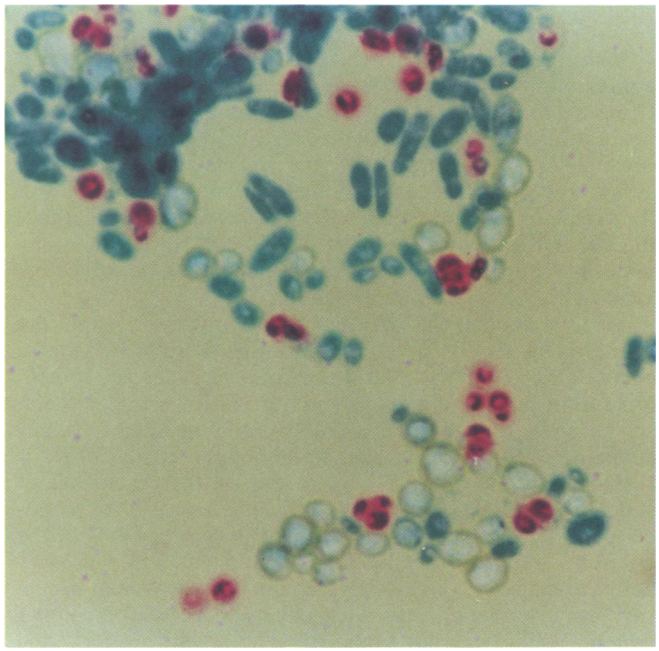

FIG. 7. Acid-fast stain of an ascosporogenous yeast, using the GSA. The ascospores stain red and the other yeast cells stain blue.

two of the four technologists had to be in the acceptable range as a second means of normalization (Table 2).

Cellular content was evaluated in the following manner. Each pair of slides, GSA and conventional, from each of the six sources was analyzed for the different types of cells on the slide. The different cell types (leukocytes, gram-positive cocci, epithelial cells, etc.) were listed for each pair of slides, but only those cell types observed by at least three of the four technologists were assumed to be on the slides.

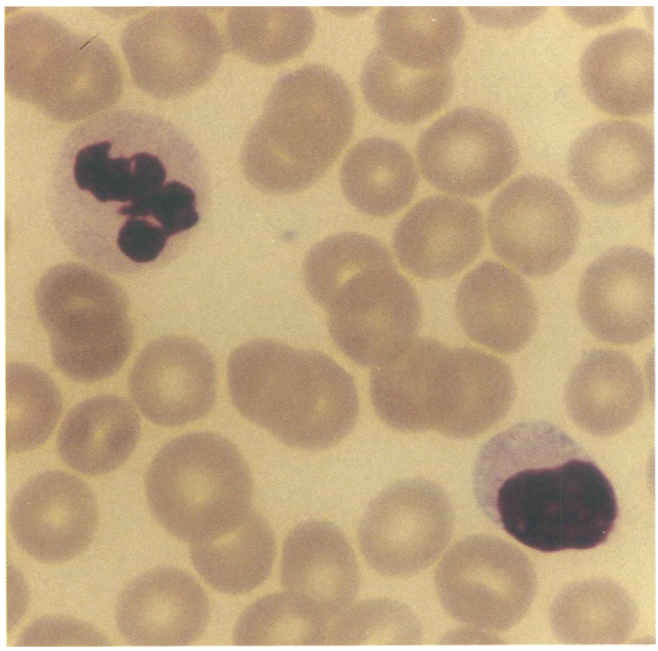

FIG. 6. Wright's stain of a blood smear, using the GSA.

Other staining procedures. Procedures for acid-fast staining were performed by using the Kinyon formula, which obviates the need for heating the carbolfuchsin. The conventional procedure is described in reference 10.

Procedures for Wright's stain require the use of Wright-Giemsa Stain Pac (no. 197-012; Curtis-Matheson Scientific). The staining reagents are made for use with automated slide stainers such as the Hema Tech Slide Stainer, but the staining technique can be easily applied to the GSA.

RESULTS

The results obtained with each staining technique demonstrated that the GSA performed as well as, and for some of the variables measured better than, the conventional method of Gram staining. In the evaluation of the quality of Gram reaction, all three sets of stained smears in phase 1 showed better results in four of the five variables when the GSA was used. A comparison of the Gram reaction readings from the two methods is presented in Fig. 4. For evaluation of decolorization, staining intensity, uniformity of stain, and background, the GSA method proved to be the more favorable of the two staining procedures. Precipitate formation was the one area in which the percent agreement was higher with the conventional method (Fig. 4). However, even with this variable, the difference in the percent agreement between the two methods was only 3%. This may be an indication that precipitate formation is a minor problem in both methods.

The data from the clinical and environmental samples again showed precipitate formation as the one area in which the GSA did not perform as well as the conventional method (Table 2). Smears from each of the six sources were examined for cellular content (Table 3). Although the results were close, the GSA proved to be slightly better than the conventional method.

DISCUSSION

All of the technologists who participated in this study had a minimum of 10 years of experience with the conventional Gram staining method, but they had little or no experience with the GSA. This suggests that even better results might be obtained as experience with the device increases. With either method, the decolorizing step is the most critical of the Gram staining procedure. By the conventional method, the decolorizing step can often be difficult to reproduce by different technologists, because it is dependent on subjective visual observation of decolorization.

An advantage of the GSA technique, however, is that the decolorizing step is a timed exposure, thereby helping to ensure reproducibility. Variations in results can, of course, arise from factors affecting the nature of the samples themselves such as age of the culture, the number of times subcultured, or, particularly, the thickness of the smear.

Because there was no way to ensure that each pair of slides from the human or environmental sources had identical material, at least 75% agreement in observations of the different cell types seen by the technologists was necessary before evaluation of cellular content. This was also necessary because some of the slide pairs had very little cellular content which was not found by some of the readers, and there was not always 100% agreement on the identity of some cells. These observations are listed in Table 3.

Analysis of the data in Table 3 reveals that, from the six different sources (throat, nasal, oral cavity, urine, clinical, and environmental) three sources had the most observations by 75% of the technologists with the GSA, two sources had the most observations with the conventional method, and one source had just as many observations with the GSA as with the conventional method. An analysis of Table 3 also reveals that the total number of sets with the highest number of observations by 75% of the technologists is almost the same (20 by GSA and 19 by conventional method). Also, there are 21 instances in which the number of observations were equal for both pairs of slides. These data suggest that the observations of cellular content were just as good with the GSA as with the conventional staining method. The rationale behind staining actual human and environmental specimens is that organic and inorganic material present in some of these specimens may affect the Gram staining process, making the stained smears more difficult to read or interpret.

In addition to performing Gram stains, the GSA can also be used to perform Wright's stains for blood smear analysis (see Fig. 6) and standard as well as modified acid-fast stainings for the detection of *Mycobacterium* and *Nocardia* species or to stain the asci of ascosporogenous yeasts (see Fig. 7). The quality of GSA staining capacity is illustrated in Fig. 5 to 7).

Not only is the GSA versatile, but, unlike other staining systems that are too heavy, too large, or require external power and utilities to operate in microgravity environments (2, 4, 6, 7), it is also self-contained, portable, lightweight, and easy to use. All testing of the GSA staining device suggests that it is an efficient and reliable apparatus for support of the microbiological diagnostic capabilities aboard the Space Station Freedom.

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